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UNITED STATES PUBLIC HEALTH SERVICE
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THE STANDARDIZATION OF DIGITALIS

A COMPARATIVE STUDY OF SOME OF THE METHODS OF ASSAYING DIGITALIS, WITH A DESCRIPTION OF AN IMPROVED MODIFICATION OF THE ONE-HOUR FROG METHOD

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SUPPLEMENT No. 52
TO THE
PUBLIC HEALTH REPORTS

WASHINGTON
GOVERNMENT PRINTING OFFICE
1925

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Introduction

This work was undertaken in cooperation with the International Conference on Biological Standards of the Health Committee of the League of Nations. At its meeting in Edinburgh in 1923 the conference adopted a resolution that an investigation be made of the several methods of digitalis assay in vogue with a view to determining the relative values obtained by these methods with certain samples of digitalis leaf. The ultimate aim of the investigation was to provide a basis for an international unit of measuring activity of preparations of the digitalis group in order that they might be adjusted to a definite and uniform standard of activity.

Three samples of digitalis leaf to be used in this investigation were submitted to the conference by Professor Magnus of Utrecht. These included a "specially prepared sample of dried digitalis leaves, preserved with precautions against access of moisture or oxygen," [1] which was to be used as a standard. It will be evident to the reader that the ouabain (crystalline strophanthin of Thoms) standard, which is at present official in the U. S. Pharmacopoeia, is not regarded as satisfactory by the conference. This attitude seems to be justified on the basis of the work of Gottlieb (1), Baker (2), Sollmann, Mendenhall, and Stingel (3), Hatcher (4), Roth (5), and others. Briefly stated, the work of these authors shows, directly or indirectly, that the rates of absorption, elimination, and destruction of different digitalis bodies, following their injection into the lymph sac of the frog, vary widely under different conditions, and with no definite relation to one another. At best, only similar preparations can therefore be compared by the frog method. Thus, ouabain, it is held, which may properly be taken as a standard for the evaluation of the activity of strophanthus preparations, is not suitable for the standardization of digitalis preparations. Furthermore, Baker (2) has shown that the toxicity of digitalis for the frog's heart at different temperatures does not follow the toxicity curve of ouabain as obtained by himself and later by Sollmann, Mendenhall, and Stingel (3). However, that this is probably due to irregular absorption of digitalis in the frog and not to some fundamental difference in action of digitalis and ouabain will be shown in the pages to follow. Evidence will also be given to show that many of the irregularities heretofore encountered in the

[1] Quoted from a preliminary report by Dr. H. H. Dale on the results of the work on digitalis standardization undertaken by the International Conference on Biological Standards, January, 1925.

bio-assay of digitalis by the frog method, such as the wide seasonal variations in susceptibility, can be accounted for by irregularity of absorption. These experiments, we believe, will indeed lessen the validity of some of the objections raised heretofore against the use of ouabain as a standard for digitalis assay.

Experimental

Preparations of three samples of powdered digitalis leaves designated "standard," "A," and "B," respectively, were made by extraction for twenty-four hours with absolute ethyl alcohol in a Soxhlet apparatus on the water bath, as described by Joachimoglu (6). This author seems to have shown that, by the above method all of the available activity in the leaf is completely extracted. The extracts were adjusted to such strength that each cubic centimeter represented the alcohol-soluble constituents of 20 mgs. of the dried leaf, and were stored in brown glass bottles at room temperature.

Two sets of extracts were prepared in the course of this work. In the first set, extraction was carried out upon an electric hot plate covered with asbestos sheet. In the second set the extraction was carried out upon the water bath at the temperature of boiling alcohol. Most of the experimental work was done with the second set of extracts, but a sufficient number of experiments upon cats were also made with the first set to indicate that the activity of this set ran somewhat lower than that of the second, as will appear from Table 1. It seems likely that the heat of the electric hot plate may have caused some injury to the active glucosides. Attention is called to this matter to suggest that should the digitalis powder eventually be accepted as a standard, its extraction will have to be carried out with a great deal of care and precision.

The results of this work include assays of the alcoholic extracts of the three digitalis powders by the cat method of Hatcher and Brody (7) with the modification as suggested by Professor Magnus; by the colorimetric method of Knudsòn and Dresbach (8); and by the frog method. The frog experiments were made according to the well known one-hour method, which is official in the U. S. Pharmacopoeia (9); by a three-hour method as suggested in a memorandum of the International Conference on Biological Standards; and lastly by a modification of the one-hour official method as worked out by us. This last appeared to be so much of an improvement over the one-hour official method that, in order to determine its general applicability, it seemed desirable to extend its examination to several commercial digitalis products. The results therefore include a comparative study of the activity of seven commercial tinctures as determined by our modification of the frog method, by the cat method, and by the colorimetric method.

ASSAY OF SAMPLES 'STANDARD," "A" AND "B"

1. THE CAT METHOD

Measured amounts of the alcoholic extracts of the respective digitalis powders were evaporated on the water bath to remove the alcohol. The syrupy residue was taken up in physiologic salt solution, each cubic centimeter to contain the equivalent of 10 mgs. of digitalis powder. After having been stirred thoroughly this was passed through a coarse folded filter paper, being then ready for infusion. That none of the activity is lost by this procedure will be shown later. (See pp. 20–21.) The solution thus prepared is injected slowly from a burette into the femoral vein of a cat of known weight kept under light ether anesthesia [1] and under artificial respiration. The infusion is continued until the heart stops beating.[2] The cat unit is expressed in terms of mgs. of drug per kilogram of body weight required to paralyze the heart. Table 1 shows the values of the three preparations as obtained by this method. A difference of from 10 to 20 per cent in activity of the two sets of extracts is shown, the probable reason for which has already been discussed.

TABLE 1.—*Assay of two sets of alcoholic extracts of samples "standard," "A," and "B" by the cat method*

Sample	First set of extracts					Second set of extracts				
	Number of experiment	Sex	Weight, kilos	Cat unit milligrams per kilo	Average	Number of experiment	Sex	Weight, kilos	Cat unit milligrams per kilo	Average
"Standard"	1	M	3.8	99	119	1	M	2.8	87	94
	2	F	1.7	140		2	M	1.7	95	
	3	F	2.5	116		3	F	1.5	97	
	4	M	1.4	120		4	F	2.4	96	
"A"	1	M	2.2	125	141	1	F	2.5	128	129
	2	F	2.2	185		2	M	2.5	140	
	3	F	2.5	128		3	M	2.5	100	
	4	F	2.4	149		4	M	1.8	148	
	5	M	3.6	124						
	6	F	2.5	137						
'B"	1	F	2.0	125	116	1	M	3.8	114	97
	2	F	1.7	120		2	M	2.6	96	
	3	F	2.7	113		3	F	2.0	88	
	4	F	2.1	105		4	F	2.2	89	

[1] Anesthesia was induced with a minimal amount of struggling, by placing the animal in a specially constructed cat box (14).

[2] This modification by Professor Magnus of the Hatcher and Brody (7) method eliminates the use of ouabain, which, together with the artificial respiration, is probably designed to meet the objection often raised against the cat method of assay on the ground that some of the digitalis bodies paralyze the respiration in the mammal. Professor Magnus's modification of the cat method makes the end point less sharp than in the original Hatcher and Brody method, and requires vigilant watching of the heart action, preferably with the aid of a stethoscope, especially in the latter part of the experiment.

From the data given in Table 1 it will be seen that on the basis of assay of the second set of extracts, the results of which are probably more reliable, "standard" and "B" are nearly equal in activity, while preparation "A" is about 30 per cent inferior.

2. THE COLORIMETRIC METHOD

The alcoholic extracts of the respective powders were concentrated on the water bath at a low temperature and made up to a suitable volume. The technique employed strictly adhered to that described by Knudson and Dresbach (8). A ouabain solution was used as standard.

The results obtained were as follows:

"Standard": 44.8 mg. per cat unit.
Sample "A": 57 mg. per cat unit.
Sample "B": 46.8 mg. per cat unit.

Taking "standard" at 100 per cent activity, samples "A" and "B" assayed at 78 and 95 per cent, respectively. There is thus very good agreement between the cat and colorimetric methods in so far as the relative activity of the three preparations is concerned, though there is lack of agreement between the two methods as concerns the absolute figures. In view of the last disparity, samples of the extracts were sent to Doctor Knudson for chemical examination. (We wish to take this opportunity to extend our thanks to Doctor Knudson for his kind cooperation.)

Doctor Knudson's findings were as follows:

"Standard": 50.0 mg. per cat unit.
Sample "A": 63.7 mg. per cat unit.
Sample "B": 52.5 mg. per cat unit.

These figures closely agree with our own, and assign exactly the same relative values for the three preparations as we had obtained.

3. THE FROG METHOD

This work was begun with the official one-hour frog method (9). The alcoholic extracts were concentrated on the water bath at low temperature, and diluted with physiologic salt solution to yield such concentrations as not to exceed 0.5 c. c. per frog of 20 to 30 grams weight. The alcohol content of the test solutions was approximately 20 to 25 per cent. Experiments were made with three different lots of frogs (*R. pipiens*) obtained during the months of June, August, and October, respectively. The results of these experiments were disappointing. We are convinced that no reliance can be placed upon results obtained from an experiment with one or two frogs on a given dose. Even by using half a dozen or more frogs on each dose the results in many cases were too variable to permit us to draw conclusions. The difficulties were, in the main, due to great variation in

individual susceptibility. Much of this seemed to have been accounted for by lack of absorption. However, that factors other than lack of absorption may be concerned in the irregular response of the frog's heart to digitalis seems to be indicated by the fact that there is no constant and uniform relationship between the presence of unabsorbed fluid in the lymph sac, the condition of the heart, and the size of the dose administered. Thus one may find the heart beating with apparently complete absorption of a dose which produces systolic standstill in the majority of frogs; and, again, the heart may be stopped by a dose less than that required to produce systolic standstill, in spite of the occasional presence of what appears to be unabsorbed fluid in the lymph sac. In some of the experiments fairly consistent results were obtained; but this was the exception rather than the rule.

As an extreme example of irregular results with the official one-hour frog method the following may be given as representing an attempt at assaying sample "A" in October:

Dose milligram per gram	Number of frogs used	Number in systolic standstill	Number with unabsorbed fluid in lymph sac	Dose milligram per gram	Number of frogs used	Number in systolic standstill	Number with unabsorbed fluid in lymph sac
0.40	6	0	0	0.80	5	2	1
.50	6	0	0	.90	6	3	1
.60	6	3	0	1.00	6	1	1
.70	6	1	5	1.20	6	2	1

Unsatisfactory experiments such as the last were frequently repeated with an extension of the observation period to three hours, but the results were no more conclusive.

A difficulty, even greater than that of encountering irregular results in any given experiment due to individual variation in absorption and, perhaps, in susceptibility, is the fact that no conclusion could be reached concerning the relative activity of the three samples examined. Even by taking into consideration only those individual assays which yielded apparently consistent results it is found that an approximate ratio of activity of the three samples obtained in June did not entirely agree with that found in August, and the two varied widely from that found in October. Thus, from our experiments in June it appeared that "standard" and "A" were nearly equal in activity, whereas "B" appeared to be weakest. The August results led us to believe that "standard" and "B" were equal, and no opinion could be arrived at concerning the value of "A," whereas the results of October pointed to "A" being about half of "standard" and "B" about 75 per cent of "standard." Clearly, it is difficult to draw conclusions from such data.

A technique of assay whereby the test solution is injected intravenously instead of in the lymph sac naturally suggested itself as a possible improvement. The idea is not entirely a new one. Hatcher and Brody (7), introducing the method of digitalis standardization by the intravenous infusion in the cat, pointed out this feature as one of the advantages of their method over the older frog lymph-sac method. Dooley and Higley (10) advocated the intramuscular route of injection in the frog to secure more uniform results, and Gottlieb (1), some years previously, studying the mechanism of digitalis poisoning on the frog's heart, injected various digitalis bodies both intramuscularly and intravenously in order to eliminate the variable factor of absorption from the lymph sac.[3]

THE INTRAVENOUS ONE-HOUR FROG METHOD OF DIGITALIS ASSAY

Our modification of the one-hour frog method, which we believe to be a great improvement, is carried out as follows:[4]

Frogs (*R. pipiens*) of 20 to 30 grams weight are used. Animals of as nearly the same weight as possible are selected for a given experiment. They are weighed in the usual manner and placed in individual cages kept in a constant temperature tank at 20° C.[5] The animals are kept at this temperature for about one hour prior to injection, and then to the end of the observation period. Twelve frogs are conveniently used in a single experiment. In the preliminary test, four graded doses, differing from one another by from 20 to 30 per cent, are administered, using three animals for each dose. From three to five minutes are required for injection, so that by the time all the animals of a series have been injected, the first of the series is due for examination of the condition of the heart. If an effective dose has been found in the preliminary series, a second group of 12 frogs, 3 each on the subeffective and effective doses, and 6 on a dose intermediate between the last two will generally suffice to complete an assay with an accuracy of about 10 per cent. The minimal dose which produces systolic standstill in the majority of the animals is taken as the minimum systolic dose.

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[3] Gottlieb's results by the intravenous method were apparently no more regular than by the lymph-sac method. Gottlieb's experiments are, however, not entirely comparable with our own for the following reasons: First, Gottlieb used alcoholic solutions for intravenous injection, which, as we shall point out later, is not permissible; secondly, the temperature does not seem to have been controlled in his experiments; and, lastly, Gottlieb's observations must have been made upon frogs with exposed hearts, as he was concerned with determining the time interval between systolic standstill and resumption of heart beat after a given dose of a digitalis preparation in order to ascertain the rate of destruction of digitalis bodies in the frog.

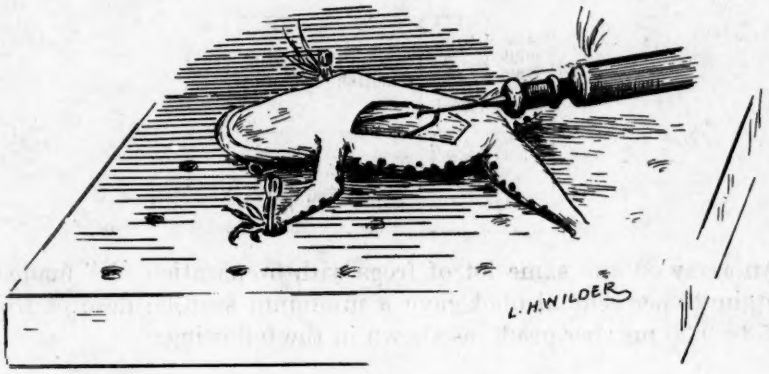
[4] After the results of this work were made known to the International Conference on Biological Standards through a preliminary report, Dr. H. H. Dale called our attention to the fact that Dr. J. H. Burn had previously used an intravenous frog method of digitalis assay, but that his results by this method did not seem better than by the lymph-sac method. It appears that Doctor Burn used a somewhat different technique in his unpublished experiments of which we knew nothing at the time this work was done.

[5] A suitable sized tank of galvanized iron is used. A few small perforations in the top allow for ventilation and an opening large enough to permit the passage of the individual frog cages is fitted with a lid. A thermometer inserted horizontally about one-half inch from the bottom of the tank registers the temperature of the water, and another inserted vertically through the top of the tank registers the temperature of the air over the water.

The test solution is prepared in the same manner as described in connection with the cat method of assay. The alcohol is removed by careful evaporation on the water bath at a temperature not over 50° C. and under a stream of cool air. The evaporation is not allowed to proceed to dryness. The small residue is taken up in physiologic salt solution to yield a concentration of from 20 to 40 mgs. of digitalis leaf to the cubic centimeter and filtered. (The average tincture would thus be diluted from 2.5 to 5 times the original volume.) The dilution is so gaged as to keep the volume to be injected between 0.02 and 0.03 c.c. per gram of frog.

The injections of the calculated doses are made into one of the musculocutaneous veins of the frog, the one on the right side having been found the more convenient.

For this purpose the brain of the frog is pithed, the animal is fastened on its back to the frog board, and a small median incision



of the skin overlying the ventral lymph sac is made. As the right flap is deflected with a fine pair of forceps the right musculo-cutaneous vein running from the skin flap up and superficially in the abdominal wall, comes into prominence. (See accompanying illustration.) This vein joins the brachial to form the subclavian which empties into the precaval vein and thence into the heart. This, as far as we know, is the only easily accessible vein that empties direct into the heart. The abdominal vein receiving the blood from the vein on the ventral surface of the hind leg drains into the liver, and the sciatic vein on the dorsal aspect of the hind leg drains into the renal-portal vein, which breaks up into a capillary network in the kidney ([11]).

The test solution is injected slowly into the musculocutaneous vein with a 26 gage needle attached to a calibrated tuberculin syringe. At the completion of the injection, and upon withdrawing of the needle from the vein, the latter is pinched for a minute or two with a small artery clamp. The animal is then taken off the board and at once returned to the constant-temperature tank. With proper technique there should be no bleeding whatever throughout the whole operation.

At the end of one hour from the time of injection, the cord is pithed, the heart is exposed in the usual manner, and its condition is examined.

Alcohol in the test solution is not permissible in this method of assay, as the results are too high and somewhat more irregular. This is shown by the following experiment:

In November, 1924, sample "B" was assayed by our method, using an alcohol-free solution. The minimum systolic dose was 0.45 mg. per gram, as shown below:

Dose, milligram per gram	Number of frogs used	Result (+=systolic standstill; −=beating)
0.30	4	+ − − −
.40	7	+ − − − − − −
.45	5	+ + + + −
.50	6	+ + + + + +

An assay on the same lot of frogs with preparation "B" made to contain 20 per cent alcohol gave a minimum systolic dose of from 0.25 to 0.30 mg. per gram, as shown in the following:

Dose, milligram per gram	Number of frogs used	Result (+=systolic standstill; −=beating)
0.20	4	+ − − −
.25	5	+ + + − −
.30	6	+ + + + + −
.40	3	+ + +
.50	3	+ + +

This discrepancy between an alcohol-containing solution and an alcohol-free solution might, of course, be interpreted to mean that the alcoholic solution contains a fraction of activity of the leaf which is not contained in the alcohol-free solution. That this is not the case is shown by an experiment in which an alcohol-free solution of sample "standard" was prepared in the usual manner, filtered, and to the

filtrate alcohol added to make 25 per cent. An assay carried out with such a solution in February, 1925, gave a minimum systolic dose of 0.20 mg. per gram or less, as shown in the experiment below:

Dose, milligram per gram	Number of animals used	Result (+=systolic standstill
0.20	3	+ + +
.30	3	+ + +
.35	3	+ + +

The minimum systolic dose of sample "standard" for this lot of frogs was 0.35 mg. per gram. (See Table 3.)

Further evidence will be cited later in connection with experiments upon cats to show that none of the activity is lost when a saline extract is made of an alcoholic preparation of digitalis from which practically all the alcohol has been removed by evaporation.

The possibility of nonspecific inorganic salts, especially potassium salts, interfering with the test was considered. The ash content of digitalis leaf according to various authorities is about 10 per cent. The combined sodium and potassium content of the ash as oxides, according to Rogers and Newcomb (12), is about 31 per cent. Assuming that all this is potassium, and that all of the available potassium in the leaf goes over into the official alcoholic preparations of the leaf, we estimated that the test solution could by no means contain more than the equivalent of 5 mgs. of potassium chloride to the cubic centimeter, and in reality would not contain more than a small fraction thereof, if any. Accordingly, experiments were made to determine the effects of potassium chloride on the frog's heart when injected intravenously either alone or in combination with a digitalis preparation.

Potassium chloride injected intravenously into the frog in physiologic salt solution has no permanent action on the heart, even in such enormous doses as 200 mgs. per kilo. When the heart is exposed shortly after the injection (5 to 15 minutes), it may be found in diastolic standstill after doses of 150 mgs. per kilo or over. The heart recovers from this, however, for, if examined at the end of an hour, 200 mgs. per kilo was without effect.

To test the influence of potassium chloride upon the reaction of the frog's heart to the intravenous injection of digitalis, experiments were made to determine the minimum systolic dose of sample "standard" made with physiologic salt solution containing varying

amounts of potassium chloride. The results of the assays were not affected by the presence of potassium chloride up to the extent of 0.5 per cent in the test solution. For illustration may be cited the following experiment made upon a lot of frogs in January, 1925. The test solution was sample "standard" in physiologic saline containing 5 mgs. potassium chloride to the cubic centimeter, The minimum systolic dose was 0.40 mg. per gram as shown below.

Dose, milligram per gram	Number of frogs used	Result (+=systolic standstill; −=beating)
0.30	4	+ − − −
.35	4	+ − − −
.40	4	+ + + +

The minimum systolic dose of "standard" for this lot of frogs without the addition of potassium chloride was precisely the same, as shown in Table 3.

When the potassium chloride content of the test solution was increased to 1 per cent the results became more irregular, and an increased susceptibility of the frog's heart to digitalis was noted.

ASSAY OF SAMPLES "STANDARD," "A," AND "B" BY THE INTRAVENOUS ONE-HOUR FROG METHOD

The activity of the alcoholic extracts of the three samples was estimated by this method on three separate lots of frogs, with complete uniformity of results. The details are given in Table 2. The figures given in this and in the subsequent tables, 3, 4, and 6, are condensed to include only those that are close to the minimum systolic dose. It is evident from the data given in Table 2 that samples "standard" and "B" are very nearly equal in activity, whereas sample "A" is about half the activity of the other two. If "standard" be taken at 100 per cent, the relative activity of the three preparations as obtained on three different lots of frogs may be summarized as follows:

Month	Sample		
	"Standard"	"A"	"B"
August	100	[1]	90.
October	100	53	91.
November	[1] 100	50	90 (estimated).

[1] Not tested.

TABLE 2.—*Assay of samples, "standard," "A," and "B" by the intravenous one-hour frog method*

Month	"Standard"				"A"				"B"			
	Dose, milligram per gram	Number of frogs used	Result [1]	Minimum systolic dose	Dose, milligram per gram	Number of frogs used	Result [1]	Minimum systolic dose	Dose, milligram per gram	Number of frogs used	Result [1]	Minimum systolic dose
August	0.40	3	− − −	0.45					0.40	6	+ − − − − −	0.50
	.45	3	+ + −						.45	3	− − −	
	.50	6	+ + + + + −						.50	6	+ + + + + −	
									.60	6	+ + + + + +	
October	0.40	6	− − − − − −	0.50	0.60	6	+ + − − − −	0.95	0.40	4	+ − − −	0.55
	.45	6	+ + − − − −		.70	3	− − −		.50	6	+ + − − − −	
	.50	6	+ + + + + +		.80	3	− − −		.55	6	+ + + + + −	
					.85	3	+ − −		.60	6	+ + + + + −	
					.90	6	+ + − − − −					
					.95	5	+ + + + −					
					1.00	6	+ + + + + −					
November					0.80	6	+ − − − − −	0.85	0.30	4	+ − − −	0.45
					.85	6	+ + + + + −		.40	7	+ − − − − − −	
					.90	6	+ + + + + −		.45	5	+ + + + −	
					1.00	4	+ + + +		.50	6	+ + + + + +	

[1] + =heart in systolic standstill; − =heart beating.

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Summarizing the results obtained by the three methods of assay the relative activity of the three samples of digitalis powder may be expressed as follows:

Method	Sample		
	"Standard"	"A"	"B"
Intravenous frog method	100	52	90
Cat method	100	73	97
Colorimetric method	100	78	95

There is thus close agreement by the several methods as regards the relative activity of "standard" and "B." The agreement is not so good when a comparison is made of the three methods as to the relative activities of "A" and "standard."

SEASONAL VARIATIONS IN SUSCEPTIBILITY OF THE FROG TO DIGITALIS

Many statements occur in the literature to the effect that the frog's heart varies greatly in its response to digitalis poisoning at different seasons of the year. The summer frog is said to be most resistant to digitalis poisoning. The seasonal variations as recorded in the literature range from 30 to 40 per cent to several hundred per cent (1). Since the factor of variable absorption has for the most part never been taken into account before, it is not altogether certain whether the marked variations in susceptibility at different seasons of the year might not be due in a large measure to irregular absorption rather than to some intrinsic peculiarities of the heart muscle or in the manner of destruction and elimination of the poison. It will be of interest, therefore, to record the observations made in the course of this work with a view to determining the minimum systolic dose of the alcoholic extract of the "standard" digitalis powder upon intravenous administration to six different lots of *R. pipiens* at different seasons of the year. These results are presented in Table 3. It will be seen that 0.35 and 0.50 mg. per gram represent the minimal and maximal effective doses, respectively, thus showing a maximum variation in susceptibility of about 30 per cent. The extreme variations in susceptibility noted heretofore must have been due to irregularities in absorption.

TABLE 3.—*Seasonal variations in susceptibility of frog's heart to digitalis. Intravenous one-hour method of assay. Alcoholic extract of "standard"*

Month	Dose, milligram per gram	Number of frogs used	Result [1]	Minimum systolic dose
1924				
August	0.30	3	− − −	0.45
	.40	3	− − −	
	.45	3	+ + −	
	.50	6	+ + + + + −	
	.60	3	+ + +	
October	0.30	3	− − −	0.50
	.40	6	− − − − − −	
	.45	6	+ + − − − −	
	.50	6	+ + + + + +	
1925				
January	0.30	3	− − −	0.40
	.35	6	− − − − − −	
	.40	7	+ + + + + + −	
	.50	4	+ + + +	
February	0.25	6	− − − − − −	0.35
	.30	6	− − − − − −	
	.35	8	+ + + + + + − −	
	.40	8	+ + + + + + + −	
	.45	8	+ + + + + + + −	
March	0.30	4	− − − −	0.45
	.40	7	+ + + − − − −	
	.45	6	+ + + + − −	
	.50	6	+ + + + + +	
April	0.40	4	+ − − −	0.45
	.45	4	+ + + +	
	.50	4	+ + + +	

[1] + = heart in systolic standstill; − = heart beating.

THE INFLUENCE OF TEMPERATURE ON THE SUSCEPTIBILITY OF THE FROG TO DIGITALIS

In 1915, Sollmann, Mendenhall, and Stingle (3) reported an important series of observations on the susceptibility of the frog's heart to ouabain as influenced by temperature. Their results indicated a progressively increasing susceptibility to ouabain poisoning with increasing temperature. Baker (2) had made similar observations in 1912 with reference to ouabain, strophanthus, and digitalis. The influence of temperature on the toxicity of strophanthus was found by Baker to be of the same order as that of ouabain, and his experiments with the latter agree very well with the observations of Sollmann, Mendenhall, and Stingle (3). Baker was, however, unable to demonstrate a similar relationship between toxicity and temperature for digitalis leaf. This, if true, is important, for it would indicate a fundamental difference in action of digitalis and ouabain in the frog, and this would constitute a valid argument against the soundness of using ouabain as a standard for the bio-assay of digitalis. With a method at hand which has given uniform results by eliminating the variable factor of absorption it seemed desirable to reinvestigate this question, especially in view of the important

bearing the results might have upon the choice of the standard for digitalis assay. Accordingly, experiments were made with the alcoholic extract of the "standard" sample of digitalis leaf to determine the minimum systolic dose by the intravenous frog method, the assays being carried out at different, but constant, temperatures.

TABLE 4.—*Effect of temperature on the susceptibility of the frog's heart to digitalis. Intravenous one-hour method of assay at different temperatures. Alcoholic extract of "standard"*

Temperature of assay	Dose, milligram per gram	Number of frogs used	Result [1]	Minimum systolic dose
15° C.	0.30	4	− − − −	[2] 0.65 (0.74)
	.40	4	− − − −	
	.50	4	− − − −	
	.60	6	+ − − − − −	
	.65	6	+ + + + − −	
	.70	6	+ + + + + −	
20° C.	0.35	6	− − − − − −	0.40
	.40	7	+ + + + + + −	
25° C.	0.20	8	− − − − − − − −	0.30
	.25	8	+ + + − − − − −	
	.30	8	+ + + + + + − −	
30° C.	0.15	8	+ − − − − − − −	0.25
	.20	8	+ + + + − − − −	
	.25	8	+ + + + + − − −	
37° C.	0.06	5	+ − − − −	0.08
	.07	5	+ + − − −	
	.08	10	+ + + + + + + − − − −	
	.10	8	+ + + + + + + + − −	
	.15	4	+ + + +	

[1] + = heart in systolic standstill; − = heart beating.
[2] This experiment was carried out on the February lot of frogs, for which the minimum systolic dose was 0.35. (See Table 3.) The figure 0.74 is obtained by making the necessary correction on the basis of the January lot of frogs, upon which the remainder of the experiments were made.

The results of these observations, which are presented in Table 4, clearly show that the susceptibility of the frog's heart to digitalis increases with temperature. Furthermore, the increased toxicity of digitalis with increasing temperature shows a very close parallelism to the increased toxicity of ouabain with increasing temperature as shown by the earlier workers (2, 3). The parallelism between digitalis and ouabain toxicity as influenced by temperatures ranging from 15 to 30° C. is shown by the curves in the appended chart. Curve B, taken from Sollmann, Mendenhall, and Stingle (3), shows the temperature effects on ouabain toxicity; curve A was constructed from figures given by Baker (2) on ouabain toxicity as influenced by temperature changes; and curve C represents our findings given in Table 4 on the influence of temperature on digitalis toxicity. All the curves are of the same parabolic type, and are nearly parallel.

All curves are of the same general type and nearly parallel.

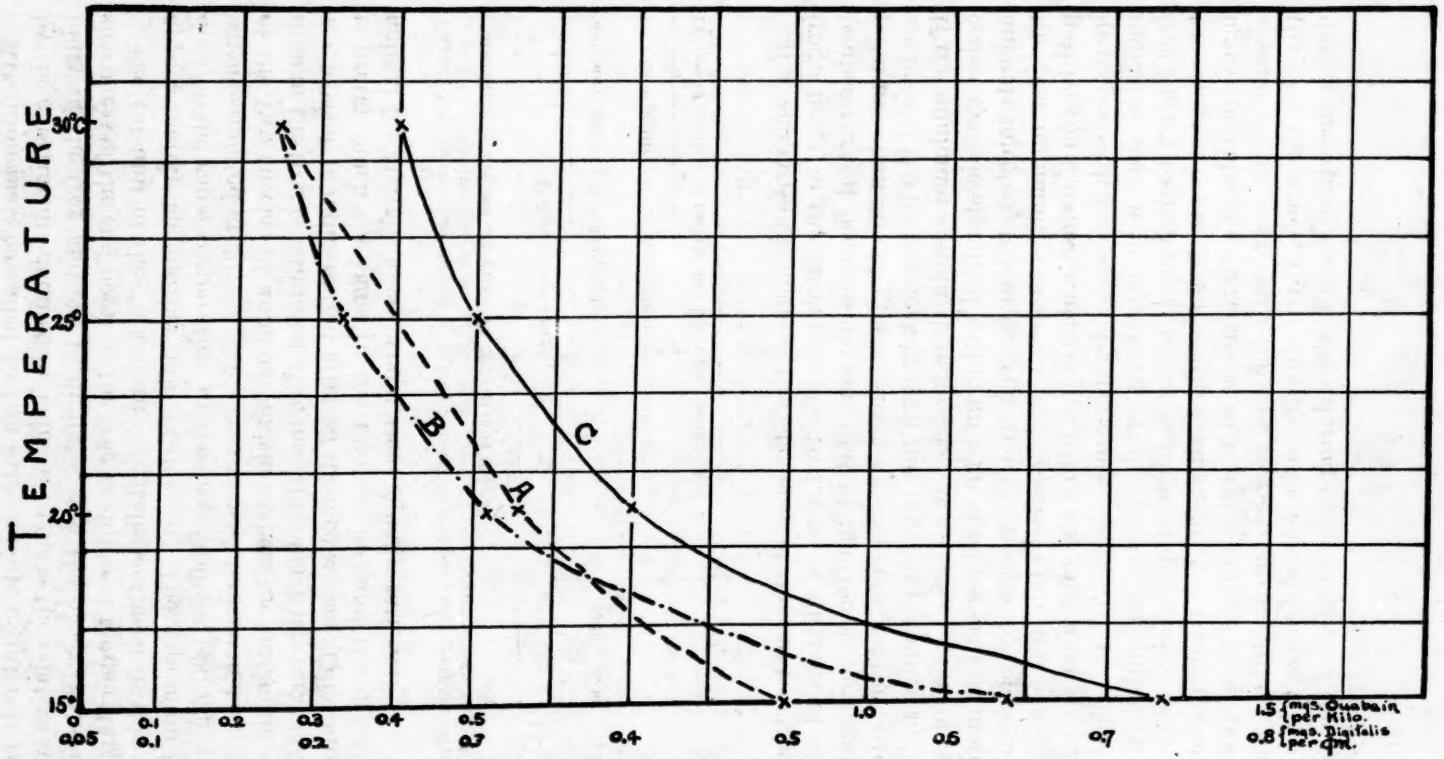

Chart showing relative susceptibility of frog's heart to digitalis and ouabain as influenced by temperature.
Curve A represents the effect of temperature on the minimum systolic dose of ouabain as given by Baker (2).
Curve B, reproduced from Sollmann, Mendenhall, and Stingle (3), shows the temperature effect on the minimum systolic dose of ouabain.
Curve C is constructed from figures given in table 4 of this paper to show the influence of temperature on the minimum systolic dose of digitalis as estimated by the intravenous method of assay.
All curves are of the same general type and nearly parallel.

Attention may be called to two other points of interest revealed by the experiments detailed in Table 4. One is the relative susceptibility of the frog and cat to digitalis, and the other is the relative sharpness of the end point in the frog at different temperatures.

The cat unit of the "standard" digitalis leaf used in this work is approximately 90 mg. per kilo (Table 1). The minimum systolic dose of the same preparation in the frog is about 400 mg. per kilo when estimated at 20° C. This enormous difference in susceptibility of the two species of animals vanishes when the minimum systolic dose in the frog is estimated at mammalian body temperature. This would seem to indicate that after all the mechanism of digitalis action is pretty much the same in the two species, and the use of either animal for digitalis assay would seem equally rational.

TABLE 5.—*Shows susceptibility of frog's heart to digitalis at different temperatures, and the relative sharpness of the end point at the temperatures indicated. An analysis of the results given in detail in Table 4*

Temperature of assay	15° C.	20° C.	25° C.	30° C.	37° C.
Minimum systolic dose, milligrams per gram	0.74	0.40	0.30	0.25	0.08
Per cent of positives on the just minimum systolic dose	67	85	75	62	60
Per cent of positives on the dose just below the minimum systolic dose	16	0	37	50	40

If a comparison is made of the sharpness of the end reaction of the frog's heart to the minimum effective dose of digitalis at different temperatures it becomes obvious that neither lower nor higher temperatures than 20° C. give as sharp an end point as the latter. An analysis of the figures dealing with this point is presented in Table 5. If the minimum systolic dose be taken in all cases as that dose which causes systolic standstill within an hour in 60 per cent or more of the animals injected, it will be seen that the percentage of positives on the just minimum systolic dose decreases, while the percentage of positives on the dose, which is just below the minimum systolic dose, increases with increasing temperatures above 20° C. This clearly indicates that with increasing or decreasing susceptibility of the frog's heart to digitalis at higher or lower temperatures, respectively, the reaction is also less uniform than at 20° C. Examination of the figures in Table 3 with reference to sharpness in end point at different seasons of the year, with the test carried out at 20° C., will show that, on the whole, there appears to be less irregularity in reaction during the winter months than during other months of the year.

A COMPARISON OF THE ACTIVITY OF COMMERCIAL DIGITALIS PRODUCTS ASSAYED BY THE CAT, THE COLORIMETRIC, AND THE INTRAVENOUS FROG METHODS

Seven tinctures of digitalis, six commercial and one that had been prepared at the University of Minnesota, were submitted to assay by the three methods indicated, in order to assure ourselves of the general applicability of our modification of the one-hour frog method. The technique of the three methods of assay was the same as that described in the earlier part of the paper.

Following are brief descriptions of the tinctures examined:

No. 1. A tincture of *Digitalis lutea*, made by the U. S. P. process and physiologically standardized by the Hatcher cat method. Cat unit = 0.65 c. c.

No. 2. Tincture digitalis, made by the U. S. P. process from the leaf of *Digitalis purpurea*, and physiologically standardized by the Hatcher cat method. Cat unit given at 0.65 c. c.

No. 3. Tincture digitalis U. S. P. IX, made at the College of Pharmacy of the University of Minnesota in December, 1918, from the leaves of *Digitalis purpurea*. This tincture carried a potency certificate dated January, 1919, giving an activity of 0.60 c. c. per cat unit.

No. 4. Tincture digitalis (fat free) manufactured in November, 1924, and advised not to be used after 12 months from date of manufacture. The tincture is stated to have been standardized by physiologic methods and labeled 150 per cent U. S. P. standard.

No. 5. A fat-free tincture stated to have been physiologically tested and standardized January 8, 1925.

No. 6. Tincture digitalis. Physiologically assayed by the U. S. P. method and standardized.

No. 7. Tincture digitalis U. S. P. IX. Physiologically tested.

The first of the seven tinctures was assayed by the intravenous frog method on the February lot of frogs, and the remaining six on a lot of frogs received in March, 1925. The minimum systolic dose of the "standard" digitalis powder for the February lot of frogs was 0.35 mg. per gram, while for the March lot it was 0.45 mg. per gram (Table 3). The results of the assays by the intravenous frog method are given in Table 6. The potency of the tinctures examined is expressed in terms of mgs. of "standard" digitalis powder per cubic centimeter of tincture.

The results of the assays by the cat method are given in Table 7. The potency of the tinctures in this case is also expressed in terms of milligrams of "standard" digitalis powder per cubic centimeter of tincture, in order to afford an easy means of comparing the frog results with the cat results. A glance at the figures of each of the

TABLE 6.—*Assay of seven tinctures of digitalis by the intravenous one-hour frog method*

Preparation	Dose, cubic centimeter per gram	Number of frogs used	Result [1]	Minimum systolic dose, cubic centimeter per gram	Minimum systolic dose of "standard" mg. per gram	Activity, milligrams of "standard" per cubic centimeter of tincture
No. 1	0.0030	3	− − −	0.004	0.35	85
	.0035	6	+ + − − − −			
	.0040	6	+ + + + + −			
	.0050	3	+ + +			
No. 2	0.0070	4	− − − −	0.0090	0.45	50
	.0080	6	+ + + − − −			
	.0085	4	+ − − −			
	.0090	6	+ + + + − −			
No. 3	0.0050	4	− − − −	0.0060	0.45	75
	.0060	4	+ + + −			
	.0070	4	+ + + +			
No. 4	0.0045	4	+ − − −	0.0050	0.45	90
	.0050	6	+ + + + − −			
	.0055	6	+ + + + + +			
	.0060	6	+ + + + + +			
No. 5	0.0070	6	+ − − − − −	0.0110	0.45	41
	.0080	5	− − − − −			
	.0090	4	− − − −			
	.0100	4	+ + − −			
	.0110	4	+ + + +			
No. 6	0.0050	4	+ + − −	0.0060	0.45	75
	.0060	4	+ + + −			
	.0065	4	+ + + +			
No. 7	0.0055	4	− − − −	0.0065	0.45	69
	.0060	4	− − − −			
	.0065	4	+ + + −			
	.0070	4	+ + + +			

[1] + =systolic standstill; − =beating.

last columns of Tables 6 and 7 will show that the agreement of the two methods of assay is not as good as might be desired. The discrepancy, wherever it occurs, is always in the same direction, the values being higher by the cat method than by the frog method. It is interesting that the discrepancy noted in the earlier part of the work in connection with the examination of the three samples of digitalis leaf, "standard," "A," and "B," was of the same character.

We have no explanation to offer for this discrepancy. It is not a very marked one, and were it not for its constant occurrence in the same direction it might be regarded as possibly within the limits of error of either method.

TABLE 7.—*Assay of seven tinctures of digitalis by the cat method*

Preparation	Experiment No.	Sex	Weight, kilos	Cat unit in cubic centimeters of tincture	Average	Activity, milligrams of "standard" per cubic centimeter of tincture
No. 1	1	F	1.9	0.66	0.77	122
	2	M	2.5	1.05		
	3	M	2.4	.63		
	4	F	3.4	.54		
	5	F	3.0	.96		
	6	F	2.9	.81		
No. 2	1	M	3.0	1.64	1.76	53
	2	M	3.7	1.57		
	3	M	3.7	2.26		
	4	M	3.7	1.56		
No. 3	1	F	3.0	0.83	1.00	94
	2	F	2.0	*.87		
	3	M	1.9	1.43		
	4	F	2.5	.86		
No. 4	1	F	1.8	0.92	0.87	108
	2	F	2.8	.77		
	3	F	2.5	.92		
No. 5	1	F	2.7	2.08	2.26	41
	2	F	2.1	2.48		
	3	F	2.7	2.22		
No. 6	1	M	3.6	1.13	1.08	87
	2	F	2.3	1.09		
	3	F	1.8	1.02		
No. 7	1	F	2.4	0.98	1.01	93
	2	F	3.1	1.10		
	3	M	3.7	.94		

* This cat had received the water insoluble residue of 20.0 c. c. of tincture No. 2, showing that the residue is devoid of any activity. For a detailed discussion see text.

The activity of the seven tinctures assayed by the colorimetric method is given below. The figures represent the average of duplicate experiments, which agreed very closely.

Number of tincture	Mg. per cat unit	Number of tincture	Mg. per cat unit
1	42.7	5	92.7
2	87.5	6	53.5
3	52.0	7	59.5
4	49.0		

The relative activities of the whole group of preparations examined by the three methods, "standard" being taken at 100 in each case, may now be given for convenience of comparison, as follows:

Preparation	Cat method	Intravenous frog method	Colorimetric method
"Standard"	100	100	100
Sample "A"	73	53	78
Sample "B"	97	90	95
Tincture No. 1	122	85	105
Tincture No. 2	53	50	51
Tincture No. 3	94	75	86
Tincture No. 4	108	90	91
Tincture No. 5	41	41	48
Tincture No. 6	87	75	84
Tincture No. 7	93	69	75

The results of assay of the six commercial products are gratifying, in that most of them are fairly uniform in activity. Tinctures 2 and 5 are exceptions. The low activity of the latter is probably accounted for by the fact that the manufacturers of this particular tincture, so it is understood, assay their digitalis products by the guinea-pig method. If our supposition is correct, this observation emphasizes the importance of using a uniform method of assay.

Special attention should be called to preparation No. 3, the University of Minnesota tincture. This tincture was made in 1918. At the time of its preparation the cat unit of this tincture was 0.6 c. c., according to tests made at the University of Minnesota. Evidently the tincture has undergone relatively little deterioration in a period of nearly seven years. The sample, which was recently submitted to us for examination, had been kept in a brown-glass bottle, corked and paraffined. This observation is in harmony with the published results of Hatcher and Eggleston (13).

The test solution used in all our experiments, whether done by the cat method or by the intravenous frog method, has been an alcohol-free solution obtained by removing the alcohol from a measured volume of tincture or alcoholic extract on the water bath at low temperature and taking up the residue in a suitable volume of physiologic saline. A small amount of insoluble resinous material is filtered off. This is, of course, variable. In some preparations there is practically none of it, whereas in others there is an appreciable amount. In the frog method of assay it has been found essential to remove the alcohol or the results are untrustworthy, as pointed out earlier. In the experiments upon the cat, alcohol-free preparations have given us somewhat more uniform results, though slightly lower ones, than when the unevaporated tincture is injected after dilution of about 1:10 with physiologic saline. That none of the activity of the tincture is sacrificed by using alcohol-free solutions is shown by the following crucial experiments:

The water-insoluble residue of 20 c. c. of tincture No. 2, which was one of the tinctures yielding the heaviest amount of water-insoluble material, was dissolved in a small volume of alcohol, diluted with 10 volumes of physiologic salt solution, and injected slowly into the femoral vein of a cat weighing 2 kilograms. No effects whatever were noted from it. After a brief interval this was followed by the injection of test solution of tincture No. 3 in the usual manner. The cat took an amount corresponding to 0.87 c. c. of the latter tincture per kilogram, or the full cat-unit for this particular preparation (Table 7), showing conclusively that the water-insoluble residue of 20 c. c. of tincture No. 2 was, for practical purposes, devoid of any activity.

Discussion

The conclusions that can be drawn from this work seem clear. The cat method of digitalis assay is satisfactory and gives sufficiently uniform results for all practical purposes. An unusually susceptible or unusually tolerant animal is occasionally met with, and there appears to be no way of eliminating this disturbing feature, except by increasing the number of animals to a point where an average is obtained from which the maximum variation is what might be regarded as within experimental limits of error. This may require considerable time and material, but the results are reasonably uniform to enable one to estimate the potency of a digitalis preparation with a fair degree of accuracy and with considerable confidence.

The modification of the Hatcher and Brody method as suggested by Professor Magnus is suitable for digitalis preparations from which all or most of the alcohol has been removed. This modified method, which was used throughout this work, has, in our experience, not given any more uniform results than can be obtained with the original method of Hatcher and Brody wherein ouabain is used. This assertion is based on an extensive experience gained by one of us (S) with the method of Hatcher and Brody some years ago in Doctor Hatcher's laboratory. The modification is, of course, essential, should the ouabain standard be discarded, a matter to which we shall refer later.

The lymph-sac one-hour frog method of digitalis assay presents in our experience very serious difficulties. We may have been so unfortunate as to run into unusually poor lots of frogs, although the animals used appeared to be healthy and in good condition. However, even a cursory review of the literature will reveal that we are not alone in finding difficulties with this method of digitalis assay. We believe that the variable and irregular absorption of digitalis bodies from the lymph sac of the frog constitutes the greatest single factor in accounting for the extreme variation in susceptibility of the frog at the same or different seasons of the year. By making the

injections into the vein, as we do, instead of in the lymph-sac, we believe we have eliminated the most disturbing factor in the one-hour frog method; and, as our results show, by introducing this modification we have made the frog method as satisfactory, accurate, and reliable as the cat method of assay. Occasional irregularities in the intravenous frog method of assay are encountered. This is due to individual variation in susceptibility on account of variation in the rate of destruction, elimination, and, perhaps, other unknown and uncontrollable factors. Such occasional irregularities in the intravenous frog method are no more disturbing than similar irregularities met with in the cat method of assay. Finally, the relative ease with which frogs can be procured, their lower cost, and the fact that the frog method is much less time consuming than the cat method, give the former very definite advantages over the latter.

In conclusion a word must be said in reference to the contemplated standard for the bio-assay of digitalis. If we could be assured of uniformity in activity of the "standard" digitalis leaf, and of uniformly complete exhaustion of its activity by alcohol extraction in the Soxhlet apparatus as recommended by Joachimoglu (6) it would be preferable, on theoretical grounds at least, to the present ouabain standard. It must be added, however, that there appears to be no really good reason for discarding ouabain in favor of some other standard. It is a substance of definite chemical composition, having definite digitalis action, and experience has shown it to be of uniform activity. The Hatcher and Brody cat method of assay is based upon the experimental proof that ouabain replaces quantitatively the other digitalis bodies (7), and our experiments reported herein demonstrate a parallelism in the susceptibility of the frog's heart to digitalis and ouabain at different temperatures.

Summary and Conclusions

A modification of the one-hour frog method of digitalis assay, wherein the test solution is injected intravenously, is described. The uncertainties due to irregular absorption from the lymph sac are thus eliminated. Clear-cut and uniform results have been obtained by this method of assay.

Observations by this method on seven different lots of *R. pipiens* over a period of nearly one year have shown that the maximum seasonal variation in susceptibility of the frog's heart to digitalis is about 30 per cent.

The susceptibility of the frog's heart to digitalis at temperatures ranging from 15° to 37° C. was studied by the intravenous method. Complete parallelism between digitalis and ouabain was noted.

Results are presented to show the relative activity of three specially prepared samples of digitalis powder and of seven tinctures of digitalis as estimated by the cat method, the colorimetric method, and the intravenous frog method, respectively.

The bearing of these findings on the choice of method for digitalis assay and on the suitability of ouabain as a standard is discussed.

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